

# THE RÔLE OF BACTERIA IN AUTOLYZING TISSUE<sup>1</sup>

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Autolysis by definition designates a process of self-digestion and specifically refers to the dissolution of tissues by enzymes present in the tissue cells, without the aid of micro-organisms. Wells (1925) has given an extensive review of the literature on this subject, the details of which are not within the scope of this paper.

Autolysis has been widely studied in animal fluids and tissues, the general thought being that such tissues when aseptically removed are sterile. Most investigators, however, who recognize the difficulty of maintaining asepsis in autolysis experiments, have added small amounts of toluene, chloroform, or other preservative to the autolyzing digest; the idea being that the preservative, while not absolutely inhibitory to enzyme action would nevertheless prevent the growth of bacteria. Bradley and his associates (1915) in an extensive review of autolysis covering many years, record no attempt to determine the possible presence of bacteria in their preparations. Much of their work was done with animal liver incubated with chloroform water or toluene water as a preservative. Recently Herron and McEllroy (1933) report that autolysis of beef liver in N/50 HCl to which a small amount of chloroform was added as a preservative, increased the potency of the liver material in the treatment of pernicious anemia. Mapson (1932) has also reported that the autolysis of beef liver at a pH of 4.5 to 5 increased the potency

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of the liver in its effects on the growth of the young rat. There is no record in these studies of attempts to determine the presence or absence of bacterial growth in the autolyzing preparations. It is apparently taken for granted by many investigators in this field that alterations in pH or the addition of small amounts of toluene or chloroform will prevent bacterial growth.

Wolbach and Saiki (1909) have shown that anaerobic bacteria are almost always present in aseptically-removed dog livers. They mention that the presence of these bacteria may account for many of the changes occurring in so-called autolysis of aseptically-removed liver. Mueller (1916) used sodium fluoride, chloroform, tricresol, and toluene successfully in the autolysis of blood but after attempts to autolyze liver in the presence of these antiseptics concluded: "It may be said that in some of the experiments in which liver was autolyzed there was apparently so much putrefaction, in spite of reasonable amounts of preservative, that positive results would have been questioned even if obtained." Gibbons and Reed (1930) taking elaborate precautions against contamination in the removal of tissues, studied the autolysis of fish muscle and guinea-pig kidney. They report finding large gram-positive bacilli in some of their digests in spite of the precautions taken.

In our studies on the bacterial flora of aseptically-removed pieces of dog liver and muscle (Trusler and Reeves (1934)) we have found that these tissues, particularly liver, almost invariably contain bacteria. Furthermore, among the bacteria commonly found are spore-forming, anaerobic, gas-forming bacilli, whose growth is not regularly prevented by alterations in pH or by the addition of weak antiseptic solutions.

We have repeated the work of Herron and McEllroy in order to determine if bacteria will grow under the conditions of autolysis which they specify. We have also conducted simple experiments to determine if variations in pH or addition of the antiseptics commonly used in studies in autolysis would inhibit the growth of bacteria in autolyzing preparations where the livers of slaughter house animals were used.

## EXPERIMENTAL OBSERVATIONS

Herron and McEllroy (1933) report that when ground beef-liver is added to N/50 HCl in 1:5 proportion and allowed to incubate at 37.5 to 40°C. for ten days the potency of the liver in the treatment of pernicious anemia is increased 5 to 8 times. As a preservative, 25 cc. of chloroform per 1,000 grams of liver were added to the digest.

*Experiment 1*

A beef liver weighing 8.5 pounds was obtained from the abattoir and prepared as follows: 3 liters of scalding, autoclaved distilled water were poured over the surface of the liver. The liver was handled with aseptic surgical technique and ground in a sterile food chopper. 1,000 grams of the ground liver were placed in a sterile 10 liter flask. Five liters of N/50 HCl and 25 cc. of chloroform were added. The flask was thoroughly shaken and placed in the incubator at 37.5°C. to autolyze. The preparation was thoroughly shaken daily. Gross observations as to color, gas-formation, odor and appearance were made. Stained bacterial smears were made of the flask contents every other day. Determinations of pH were made of the digest at the beginning of the experiment and after ten days' incubation.

*Results*

Gross observations revealed that this concentration of HCl precipitated liver proteins and that the supernatant fluid was brown in color. The odor was never putrid or offensive. As incubation progressed it was noticed that the liver proteins became more finely divided and digested. After four days the fluid above the liver proteins cleared and in the unagitated state, appeared transparent and amber-colored.

The initial pH of the digest was in the neighborhood of 2. After ten days' incubation the pH of the flask contents was around 5.

There was an abundant growth of different kinds of bacteria in the digest. This is clearly demonstrated by photographs (plate 1) taken of smears of the flask contents stained by Gram's

method. Photograph 1 was taken after twenty-four hours' incubation. Photograph 2 was taken after ten days' incubation. Stained smears made of the flask contents after twenty-four hours' incubation revealed an occasional short-chained streptococcus and several large gram-positive bacilli.

### *Experiment 2*

Since the autolyzed liver product available commercially is prepared from hog liver, it was of interest to see if hog liver autolyzed under the conditions specified by Herron and McEllroy would allow bacterial growth.

Pig livers were obtained fresh from the abattoir, prepared and autolyzed in the same manner described in experiment 1. In this case however, 2,000 grams of hog liver were autolyzed. Ten liters of N/50 HCl and 50 cc. of chloroform were added to the digest. Contrary to our expectations no bacteria were seen in stained smears made of the autolyzing preparation. This disparity in results might be explained in either of two ways.

It is possible that this particular autolyzing preparation at no time contained bacteria. It is likewise possible that bacteria were present in the digest at the time of its preparation, but were organisms of such low resistance that growth was prevented by the conditions of the experiment.

It was of interest to note that bacteria grew in the beef-liver digest in experiment 1 but failed to grow in the hog-liver digest in experiment 2. For a further study of these observations experiment 3 was carried out.

### *Experiment 3*

For this experiment, 1 beef liver and 3 hog livers were obtained fresh from the abattoir and 3 different digests were prepared. One digest contained 2,000 grams of beef liver with 50 cc. of chloroform added as a preservative; one digest contained 2,000 grams of hog liver with 50 cc. of chloroform; and the other digest contained 1,000 grams of hog liver with 25 cc. of chloroform. The digests were prepared and incubated in the same manner as described above.

In this experiment no bacteria grew in the beef-liver digest, while large numbers of gram-positive bacilli grew in each of the hog-liver digests. Photograph 3 (plate 1) was taken of a stained smear made of the 2,000-gram digest of hog liver after four days' incubation. The bacterial flora of each of the hog-liver digests was similar morphologically.

The limited data in these experiments demonstrate no uniformity in results. In the first experiment bacteria grew abundantly in the beef-liver preparation. In the second experiment hog liver autolyzed in a similar way was free from bacterial growth. In the last experiment, however, the results were reversed; bacteria grew abundantly in each of the hog-liver digests while the preparation of beef liver was free from bacterial growth.

In the experiments of Mapson (1932) the liver was finely minced, brought to a pH of 4.5 to 5 and incubated at 37° to 40°C. for twenty-four hours. He states that under these conditions autolysis is facilitated and bacterial growth is inhibited. It seemed advisable to ascertain at what pH the growth of bacteria in digests of beef liver would be inhibited.

#### *Experiment 4*

Beef liver was prepared as described above. The ground liver was divided into 80-gram amounts and placed in 14 sterile Erlenmeyer flasks. Different solutions were made up with HCl and NaOH so that the final preparations each consisted of 80 grams of ground liver in approximately 250 cc. solutions with a pH range from 1 to 14 inclusive. The pH of the different digests was adjusted after the solutions were added to the liver so that the immediate buffering action of the liver proteins in the weaker solutions was accounted for. The flasks were thoroughly shaken and placed in the incubator at 37.5°C. to autolyze. The flasks were shaken daily and gross observations made on the lysis of the liver proteins, odor and presence of gas formation. Stained bacterial smears were made of the different digests throughout the period of incubation, namely, after 24, 48, 72, 216, and 264 hours respectively. This represented observations over a period of eleven days.

After eleven days' incubation the pH of each digest was again determined. Cultures were made using a good anaerobic culture medium (Trusler and Reeves (1934)) and the flasks with their contents were autoclaved. The preparations were then filtered and 2-cc. amounts of the filtrates were injected intravenously in a dog arranged so that kymographic tracings of blood pressure would indicate the presence of pressor amines. These amines are formed by the putrefactive action of bacteria upon animal tissue as reported by Barger and Walpole (1908-09).

An abundant bacterial growth occurred in all the preparations except the digest with an initial pH of 14. No bacteria were demonstrated in this digest at any time. Those preparations with an initial pH of 1 and 2 allowed only a limited growth of

TABLE 1

*Showing the pH of the different digests at the beginning of experiment 4 and after eleven days' incubation*

Initial pH.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
pH after 11 days' incubation.....	6.8	7.4	7.3	7.3	7.5	7.6	7.6	7.4	7.4	7.4	7.5	7.4	7.5	Still above 10

bacteria within the first twenty-four hours of incubation. After that the bacterial growth in all the digests was abundant. Morphologically there were many different kinds of bacteria present. The bacterial flora of the contents of the different flasks varied from day to day but the organisms predominating on all the smears were the gram-positive, spore-forming types. Photograph 4 (plate 1) was taken of a stained smear made of the digest with an initial pH of 1 after seventy-two hours' incubation.

Most of these preparations had a marked odor of putrefaction, but we wish to stress the fact that absence of putrid odor does not necessarily mean the absence of bacterial growth.

Cultures from all the preparations except the digest with an initial pH of 14 were positive for bacterial growth, demonstrated both grossly and by smear. The kinds of bacteria growing in

the cultures were similar to the flora growing in the original flasks.

Physiological tests of the different digests showed a marked pressor effect upon the blood pressure of the dog which was due in all probability to amines formed by the action of bacteria upon the liver protein.

TABLE 2

*Showing the presence of bacteria in beef liver digests when various concentrations of antiseptics were used as preservatives (experiment 5)*

Presence of bacteria was determined by smears stained by Gram's method. The results of cultures are discussed in experiment 5.

ANTISEPTIC	INCUBATION PERIOD IN HOURS				
	24	48	72	216	264
Chloroform water.....	+	+	+	+	+
5 per cent chloroform.....	+	+	+	+	+
25 per cent chloroform.....	+	+	+	+	+
Toluene water.....	+	+	+	+	+
5 per cent toluene.....	+	+	+	+	+
25 per cent toluene.....	+	+	+	—	—
5 per cent ethyl alcohol.....	+	+	+	+	+
25 per cent ethyl alcohol.....	+	+	+	—	—
50 per cent ethyl alcohol.....	+	—	—	—	—
0.5 per cent phenol.....	+	+	+	+	+
1 per cent phenol.....	+	+	+	+	+
5 per cent phenol.....	—	—	—	—	—

+ = vegetative forms present; and — = vegetative forms absent.

### *Experiment 5*

Since it is apparently taken for granted by many investigators that the addition of toluene, chloroform and other antiseptics in small amounts will prevent bacterial growth, it was decided to test the truth of this conception so far as a single beef liver was concerned.

Twelve digests of beef liver were prepared in the same manner as described in experiment 4. Sterile water was combined with different antiseptics so that the final solution added to the 80 grams of liver equaled 250 cc. Table 2 presents the results of this experiment. It was found that bacteria grew in all the

digests where the concentration of the preservative was not sufficient to cause thorough coagulation of the liver protein. The flasks containing only chloroform or toluene water showed gas production and other gross evidence of bacterial growth. This was noted also in some of the stronger antiseptic concentrations. Usually there were no odors of putrefaction and several of the flasks showed no gross evidence of bacterial growth; nevertheless, smears made of these digests were positive for vegetative forms of bacteria. It is true that the stronger antiseptic solutions definitely inhibited bacterial growth. It can be seen by reference to table 2 that when beef liver is autolyzed in the presence of the stronger antiseptics there is at first a bacterial growth which, upon further incubation, disappears. For example where the digests contained 25 and 50 per cent alcohol and 25 per cent toluene as preservatives, bacterial growth was demonstrated for the first days of incubation, after which the digests contained no vegetative forms of bacteria. However, when the preparations were sub-cultured into routine anaerobic culture medium after eleven days' incubation, bacteria grew in all the cultures except those taken from the flasks to which 50 per cent alcohol and 5 per cent phenol had been added as preservative. Obviously, such strong antiseptics could have no place in studies of autolysis.

#### DISCUSSION

In these simple experiments we have shown that the addition of mild antiseptics does not regularly prevent bacterial growth in autolyzing preparations of fresh beef or hog livers as they are obtained from the slaughter house. We have likewise shown that a marked alteration in the initial pH of the digest does not regularly prevent bacterial growth.

We are not in position to make a definite statement as to the source of these bacteria. It is obvious that slaughter-house livers have surface contamination with many varieties of organisms. However, we did subject these livers to repeated washings with scalding water until the surfaces of the liver were coagulated. From this point the preparations were made with sterile



technique. Although these precautions cannot insure against surface contamination it is nevertheless true that two of the digests, one of hog liver and one of beef liver did remain sterile as shown by repeated smears and a culture taken after eleven days' incubation. It is possible that the absence of bacteria in these two digests might have been due to the preservative which was added. The preservative, however, did not prevent bacterial growth in numerous other digests prepared in a similar way.

We are of the opinion that the bacteria which grew in these autolyzing preparations were lying dormant in the livers of the living animals. We think it highly probable that they are present in some livers, absent in others. We have repeatedly demonstrated their presence in aseptically removed pieces of liver and muscle taken from the living dog. Wolbach and Saiki, Reith, Berg, Zau and Jobling and others have reported their presence in the tissues of the dog and other animals. We believe that these organisms are highly resistant strains of sporulating, gas-forming, anaerobic bacilli which are saprophytic in nature. They grow readily in dead tissue, (Trusler and Reeves (1934)).

We need not discuss further the source of the bacteria which grew in our autolyzing preparations. The fact remains that there were strains of bacteria present in the digests of slaughterhouse livers which grew in the presence of preservatives unless the preservative was present in sufficient concentration to coagulate the liver proteins. Even in this latter instance bacteria grew to a limited extent. This phenomenon may be due to the fact that the bacteria grew in the center of masses of animal tissue where the preservative had not penetrated. Photograph 5 (plate 1) was taken of a stained smear made of a ground beef-liver digest which had been incubated twenty-four hours in the presence of 25 per cent toluene. After eight to ten days' incubation no vegetative forms of bacteria were demonstrated in smears made from this preparation. Nevertheless, subsequent culture of this digest was positive for bacterial growth.

It was observed in experiment 4 that, although the initial pH of the different digests ranged from 1 to 14, the pH determinations made of these preparations after eleven days' incubation

revealed that all but one of them was near the neutral point as shown in table 1. We have not as yet made sufficient study to explain this phenomenon. It seems logical however, that the change of reaction toward the neutral point may have been caused by the buffering action of degradation products resulting both from autolysis and bacterial digestion of the liver substance. It is possible that this tendency of the reaction to approach the neutral may explain the fact that spore-forming bacteria were able to grow in digests with such an initial wide range of pH.

The fact that bacterial contamination may influence the results of experiments on autolysis needs no comment. Herron and McEllroy (1933), however, have reported an interesting observation to the effect that autolysis of beef liver increases its potency in the treatment of pernicious anemia. We have shown that bacteria may grow in such liver digests without giving any gross evidence of putrefaction. It is interesting to note further that bacteria have been shown to possess an ability to synthesize vitamin B (Sunderlin and Werkman (1928)). Many reports have indicated that vitamin B may be closely related to, or associated with, the anti-anemic factor present in liver.

We wish again to emphasize the fact that any studies, involving the autolysis of animal tissue may be vitiated by growth of bacteria in the digests. Especially is this true if gross quantities of tissue emulsions are being studied. In none of our experiments did we use small quantities of finely-minced, widely-dispersed tissue suspensions. It is possible that under such conditions preservatives might be more effective. The problem is worthy of more careful investigation.

#### SUMMARY

1. Many past experiments on the subject of tissue autolysis have not given proper consideration to the possible presence of bacteria in the digests.
2. Autolyzing digests of hog and beef liver obtained fresh from the slaughter house, have been shown to contain highly resistant strains of spore-forming bacteria.
3. Alterations in pH and the use of antiseptics as preservatives

have failed to prevent the growth of bacteria in most of the autolyzing preparations we have studied.

4. In our experiments, sterility of the digests could be determined only by bacterial smears and cultures. We repeatedly found anaerobic spore-forming bacilli growing in digests which were free from putrid odors and showed no gross evidence of bacterial growth.

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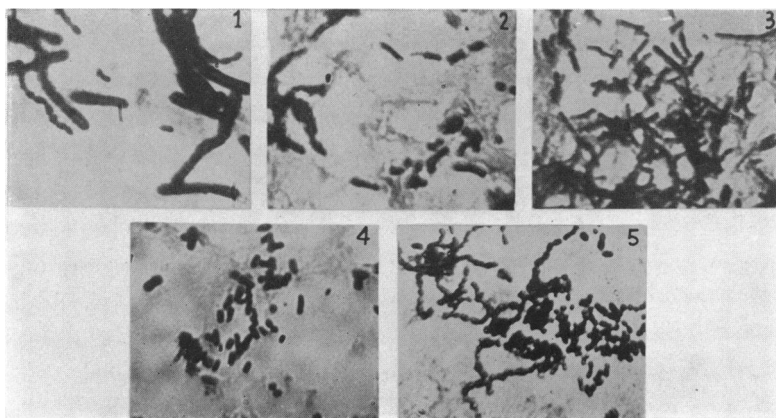
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## PLATE 1

PHOTOMICROGRAPHS OF BACTERIAL SMEARS MADE OF DIFFERENT DIGESTS OF  
LIVER AUTOLYZED UNDER VARIOUS CONDITIONS. THE SMEARS  
WERE STAINED BY GRAM'S METHOD

1. Beef liver autolyzed according to the specifications of Herron and McElroy after twenty-four hours' incubation.
2. The same preparation as 1 after ten days' incubation.
3. Hog liver autolyzed according to the specifications of Herron and McElroy after seventy-two hours' incubation.
4. Beef liver autolyzed at an initial pH of 1 after twenty-four hours' incubation.
5. Beef liver autolyzed with toluene as a preservative after twenty-four hours' incubation. The amount of toluene equaled 25 per cent of the digest.



(James R. Reeves and Hugh E. Martin: Bacteria in Autolyzing Tissue)